Comparison of Drug Resistance of Helicobacter pylori Between Children and Adults in Jilin, China

Libo Wang[1], Jianguang Zhang[2], Meilian Hu[2,3], Xiaoli Pang[1]

[1]Department of Pediatric Gastroenterology, The First Hospital of Jilin University, Changchun, Jilin, China
[2]Department of Gastroenterology, The First Hospital of Jilin University, Changchun, Jilin, China
[3]Department of Gastroenterology, Jining No.1 People's Hospital, Jining, Shandong, China

Cite this article as: Wang L, Zhang J, Hu M, Pang X. Comparison of drug resistance of *Helicobacter pylori* between children and adults in Jilin, China. *Turk J Gastroenterol.* 2021;32(12):1012-1018.

Abstract

Background: *The drug resistance of Helicobacter pylori in children is gaining more and more attention.*
Methods: *Polymerase chain reaction-reverse dot blot was used to analyze main virulence genes and drug resistance of H. pylori.*
Results: *(1) The main H. pylori vacA virulence genotypes were s1/m1 and s1/m2 in Jilin, China. There was no significant difference in H. pylori virulence genotypes between children and adults. (2) The resistance rates of H. pylori to clarithromycin, metronidazole, and levofloxacin were high, the resistance rate to tetracycline and amoxicillin were relatively low. The drug-resistance rate of clarithromycin in children was significantly higher than that in adults. The single drug-resistance rate of metronidazole in adults was significantly higher than that in children. (3) The mutation sites of clarithromycin resistance in H. pylori were mainly A2143G of 23S rRNA gene, G616A of rdxA gene in metronidazole, N81K and D91G/N/Y of gyrA gene in levofloxacin, T556S and N562D/Y of PBP1 gene in amoxicillin, AGA926-928TTC and AG926-927GT of 16S rRNA gene in tetracycline. There was significant difference in D91Y of gyrA gene in levofloxacin between children and adults. (4) In different groups, the drug-resistance rate of clarithromycin in male children was higher than that in male adults. The drug-resistance rate of clarithromycin in children with peptic ulcers was higher than that in adults.*
Conclusion: *There are some differences in drug resistance of H. pylori between children and adults, which indicated us to pay attention to the infection of H. pylori in children.*
Keywords: *Helicobacter pylori, drug resistance, gene mutation, children*

INTRODUCTION

Helicobacter pylori is a gram-negative microaerobic bacterium that can be transmitted from person to person through oral to oral or fecal to oral routes. Some indirect evidence has been reported to be transmitted through drinking water and other environmental sources.[1,2] At present, it has caused global population infection. As a developing country, China has a high infection rate of *H. pylori*, averaging about 55%.[3] Chronic active gastritis occurs in almost all patients with or without clinical symptoms after *H. pylori* infection. Studies have confirmed that the pathogenicity of *H. pylori* is mainly related to virulence factors. The expression of *H. pylori* virulence genes from different strains is different.

H. pylori infection is the main cause of chronic gastritis and peptic ulcer in children. In adults, it can cause many gastrointestinal diseases, especially gastric cancer.[4-8] *H. pylori* eradication promotes peptic ulcer healing and reduces the incidence of complications,[9] and also a primary preventive measure for gastric cancer. However, with the widespread use of antibiotics, the resistance of *H. pylori* to antibiotics has gradually increased in recent years,[10] which has become the main reason for the failure of *H. pylori* eradication. Obtaining the background of *H. pylori* resistance in the local area and comparing the differences between children and adults can guide rational drug use in the clinic, maximize the initial eradication rate of *H. pylori*, and bring practical benefits to patients.

In this study, polymerase chain reaction-reverse dot blot (PCR-RDB) was used to detect virulence genotypes and drug resistance of *H. pylori* to 5 antibiotics. To explore the background of *H. pylori* resistance in Jilin, China, and the differences between children and adults, so as to provide a reference for drug selection.

Corresponding author: ***Xiaoli Pang****, e-mail:* ***pxl0811@126.com***
Received: ***September 3, 2020*** *Accepted:* ***October 9, 2020*** *Available Online Date:* ***November 26, 2021***

DOI: ***10.5152/tjg.2021.20791***

MATERIALS AND METHODS

Ethics

All procedures performed in studies involving human participants were according to the Ethics Committee's ethical standards and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all the patients.

Participants

Collection of gastric mucosa samples from patients with gastrointestinal symptoms who received electronic gastroscopy from July to December 2019 and confirmed positive for *H. pylori* by rapid urease test (RUT). Inclusion criteria: (1) no antibiotics, proton pump inhibitors (PPI), H2 receptor antagonists, bismuth agents were used in the past 4 weeks; (2) no bleeding or perforation of gastric and duodenal lesions; and (3) no major systemic diseases or life-threatening diseases. Samples of gastric mucosa were collected and placed in the collection tube. The samples were stored at –80°C. All patients came from Jilin and signed the informed consent.

Research Methods

Nucleic Acid Extraction

Gastric mucosa samples were taken and DNA was extracted by using "nucleic acid extraction reagent" (Hangzhou Qianji Biotechnology Co., Ltd. Hangzhou, Zhejiang, China), and the specific steps were carried out according to the instruction.

PCR Amplification

Nucleic acid samples were collected and amplified using amplification reaction liquid (primer pairs were designed by Hangzhou Meilian Medical Inspection Co., Ltd. according to the sequence of *H. pylori* UreA gene, VacA gene and 5 drug-resistant genes). In the amplifier, nucleic acid samples were amplified according to the following conditions: UNG enzyme reaction at 50°C for 10 min, 1 cycle; pre-denaturation at 95°C for 10 min, 1 cycle; denaturation at 5°C for 30 s, annealing at 56°C for 30 s, elongation at 72°C for 30 s, 45 cycles, and elongation at 72°C for 5 min, 1 cycle.

Hybridization and Coloration

Membrane strips marked with sample number were put into 15 mL plastic centrifugal tube and 7-8 mL of solution A was added, then 3 tubes of PCR amplification products with corresponding to sample number were added, a centrifugal tube was heated in boiling water bath for 10 min, and then hybridized at 48°C for 1.5 h. Then the membrane strips were removed and transferred into a 50 mL centrifugal tube with preheated B liquid. It was washed gently at 48°C for 15 min, and at room temperature, the membrane strips were placed into the incubation solution and gently soaked for 30 min, and then the incubation solution was discarded. The membrane strip was immersed in the developing solution for 5-10 min, then rinsed with pure water 1-2 times to observe the results. Internal control was designed to amplify the human housekeeping gene, namely beta-globin gene with specific primers and hybridization detection with specific probes to eliminate the false negative. We use first-generation sequencing as a reference standard to compare the results obtained.

Statistical Method

SPSS 24.0 statistical software was used for analysis, and the chi-square test was used to analyze and compare the relationship between groups. $P < .05$ had a statistical difference.

Main Points

- *The drug-resistance rate of clarithromycin in children was significantly higher than that in adults. The single drug-resistance rate of metronidazole in adults was significantly higher than that in children.*
- *In different groups, the drug-resistance rate of clarithromycin in male children was higher than that in male adults; in different disease, the drug-resistance rate of clarithromycin in children with peptic ulcer was higher than that in adults.*
- *Children and adults are different in drug resistance of Helicobacter pylori. Pediatricians should pay attention to this problem.*

RESULTS

Detection Rate of H. pylori-Positive Results by PCR-DRB

Among 113 gastric mucosa samples with RUT positive, the coloration of *s1*, *s2*, *m1/m2*, UreA, and Internal Control position can be seen in 30 children and 40 adults through PCR-RDB. The positive rate in children was 68.2% (30/44), and that in adults was 58.0% (40/69).

Detection of H. pylori Virulence Gene

H. pylori virulence gene was analyzed in 30 children and 40 adults. The results are shown in Table 1. UreA gene and VacA gene were detected in all *H. pylori*-positive samples. The positive rates of VacA *s1/m1* in children

Table 1. Major Virulence Genotypes of *Helicobacter pylori* in Children and Adults

	Children		Adult	
H. pylori Virulence Genotypes	*n*	%	*n*	%
s1/m1	12	40.0	17	42.5
s1/m2	16	53.3	22	55.0
s1m1/m2	1	3.3	1	2.5
s1/s2m2	1	3.3	0	0

and adults were 44.4% (12/30) and 42.5% (17/40), and that of VacA *s1/m2* in children and adults were 53.3% (16/30) and 55.0% (22/40), respectively. The positive rates of VacA *s1m1/m2* in children and adults were 3.3% (1/30) and 2.5% (1/40), respectively. VacA *s1/s2m2* was detected in 1 child (3.3%). The *m1* and *m2* genes were generally complementary, and the *s1/m2* genotype was more than *s1/m1*. There was no significant difference in virulence genotypes of *H. pylori* between children and adults.

H. pylori Resistance to Five Antibiotics

Except that 9 strains were sensitive to 5 antibiotics in adults, and other samples with *H. pylori*-positive were resistant to antibiotics. The clarithromycin resistance rate was the highest, 86.7% (26/30) in children and 60.0% (24/40) in adults. The second was metronidazole and levofloxacin, 26.7% (8/30) and 40.0% (12/30) in children and 32.5% (13/40) and 30.0% (12/40) in adults, respectively. The resistance rates to amoxicillin and tetracycline were 13.3% (4/30) and 6.7% (2/30) in children and 5.0% (2/40) and 12.5% (5/40) in adults, respectively. The drug-resistance rate of clarithromycin in children was significantly higher than that in adults (P = .0174), but there was no significant difference in the drug-resistance rate of other antibiotics between children and adults (see Table 2 for details).

H. pylori Resistance Pattern

Single drug-resistance pattern: 9 of 40 adults with *H. pylori*-positive strains were sensitive to 5 antibiotics (22.5%) and 30 children with *H. pylori*-positive strains were all resistant to antibiotics. There were 7 clarithromycin resistance strains in children (23.3%), 5 strains in adults (16.1%); 1 amoxicillin resistance strain in children (3.3%), 1 strain in adults (3.2%); and 6 metronidazole resistance strains in adults (19.4%). Two levofloxacin resistance strains in children (6.7%). No single drug-resistant strains of tetracycline were detected. The single drug-resistance

Table 2. Resistance of *Helicobacter pylori* to Five Antibiotics (Resistance Rate)

		Number of Drug-Resistant Strains									
	Number of Patients with *H. pylori*-Positive	Clarithromycin		Metronidazole		Levofloxacin		Amoxicillin		Tetracycline	
Group		*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Children	30	26	86.7	8	26.7	12	40.0	4	13.3	2	6.7
Adults	40	24	60.0	13	32.5	12	30.0	2	5.0	5	12.5

rate of metronidazole in adults was significantly higher than that in children (P = .024).

Dual drug-resistance pattern: there were 17 strains in children and 13 strains in adults. Among them, there were 6 clarithromycin + metronidazole-resistant strains in children (20.0%), 4 strains in adults (12.9%), 8 clarithromycin + levofloxacin-resistant strains in children, and 6 strains in adults (26.7 and 19.4%), and 1 clarithromycin + amoxicillin-resistant strain in children and adults, respectively (3.3 and 3.2%); 1 clarithromycin + tetracycline-resistant strain in children (3.3%), and 2 strains in adults (6.5%); 1 levofloxacin + metronidazole strain in children accounted for 3.3%.

Multidrug resistance pattern: there were 3 triple drug-resistant strains in children, accounting for 10.0%, of which clarithromycin + cevofloxacin + amoxicillin was 2 strain, accounting for 6.7%, tetracycline + clarithromycin + metronidazole was 1 strain, accounting for 3.3%. There were 6 triple-resistant strains in adults, of which 3 were clarithromycin + metronidazole + levofloxacin, accounting for 9.7%, and 3 were clarithromycin + levofloxacin + tetracycline, accounting for 9.7%. There were no differences in dual and multi-drug-resistance rates between adults and children (see Table 3 for details).

Distribution of H. pylori *Resistance Mutation Genes*

In this study, 12 mutation phenotypes of 5 *H. pylori* resistance genes were detected, and the distribution was shown in Table 4.

Among 30 *H. pylori* resistance strains in children, 23S rRNA gene mutations were detected in all 26 clarithromycin-resistant *H. pylori* strains; the mutation sites were A2142G (3.3%) and A2143G (83.3%). Of 8 metronidazole-resistant *H. pylori* strains, G616A of the rdxA gene was the most common mutation (26.7%). Four mutation sites of the gyrA gene were detected in 12 levofloxacin-resistant *H. pylori* strains, namely N87K (20.0%), D91G (6.7%), D91Y (16.7%), and D91N (3.3%). T556S (10.0%), N562Y (3.3%), and N562D (3.3%) mutations of PBP1 gene were detected in 4 amoxicillin-resistant *H. pylori* strains. Two tetracycline-resistant *H. pylori* strains detected mutations in AGA926-928TTC (3.3%) and AG926-927GT (3.3%), respectively.

Among 31 *H. pylori* resistance strains in adults, 23S rRNA gene mutations were detected in 24 clarithromycin-resistant *H. pylori* strains, and the mutation sites were A2143G (77.4%). Of 13 metronidazole-resistant *H. pylori* strains, all mutations were G616A of the rdxA gene (41.9%). Three mutation sites of the gyrA gene were detected in 12 levofloxacin-resistant *H. pylori* strains, namely N87K

Table 3. Drug-Resistance Pattern of *Helicobacter pylori* to Antibiotics

	Group			
	Children (Total Number of Drug-Resistant Strains = 30)		Adults (Total Number of Drug-Resistant Strains = 31)	
Drug-resistant strains	*n*	%	*n*	%
Clarithromycin	7	23.3	5	16.1
Metronidazole	0	0	6	19.4
Levofloxacin	2	6.7	0	1
Amoxicillin	1	3.3	1	3.2
Tetracycline	0	0	0	0
Clarithromycin + Metronidazole	6	20.0	4	12.9
Clarithromycin + Levofloxacin	8	26.7	6	19.4
Clarithromycin + Amoxicillin	1	3.3	1	3.2
Clarithromycin + Tetracycline	1	3.3	2	6.5
Levofloxacin + Metronidazole	1	3.3	0	0
ClarithromycinL + Levofloxacin + Amoxicillin	2	6.7	0	0
Tetracycline + Levofloxacin + Metronidazole	1	3.3	0	0
ClarithromycinL + Metronidazole + Levofloxacin	0	0	3	9.7
ClarithromycinL + Levofloxacin + Tetracycline	0	0	3	9.7

Table 4. Distribution of Helicobacter pylori Resistance Mutation Genes

Mutation Sites in *H. pylori* Resistance Mutation Genes	Group			
	Children		Adults	
	n	%	*n*	%
Clarithromycin				
A2142G	1	3.3	0	0
A2143G	25	83.3	24	77.4
Metronidazole				
G616A	8	26.7	13	41.9
Levofloxacin				
N87K				
D91G	2	6.7	4	12.9
D91Y	5	16.7	0	0
D91N	1	3.3	2	6.5
Amoxicillin				
T556S	3	10.0	1	3.2
N562Y	1	3.3	0	0
N562D	1	3.3	1	3.2
Tetracycline				
AGA926-928TTC	1	3.3	1	3.2
AG926-927GT	1	3.3	4	12.9

(19.4%), D91G (12.9%), and D91N (6.5%). T556S (3.2%) and N562D (3.2%) mutations of the PBP1 gene were detected in 2 amoxicillin-resistant *H. pylori* strains. The mutation gene of 5 tetracycline-resistant *H. pylori* strains was all 16S rRNA; the main mutation sites were AGA926-928TTC (3.2%) and AG926-927GT (12.9%).

There was a significant difference in D91Y of the gyrA gene in levofloxacin between children and adults ($P = .024$).

The relationship between H. pylori *resistance and gender, disease types*

The resistance rates of *H. pylori* to 5 antibiotics in different groups were compared, as shown in Table 5.

In different gender groups, the drug resistance rates of clarithromycin in male children were higher than those in male adults ($P = .0035$). There was no significant difference in other groups.

In different disease types, the drug-resistance rate of clarithromycin in children with peptic ulcers was higher than in adults ($P = .0053$). There was no significant difference in other groups. Non-ulcer dyspepsia includes gastritis and gastric polyp.

DISCUSSION

H. pylori infection can cause chronic progressive gastritis and various diseases, including stomach ulcer, duodenal ulcer, and gastric cancer. It has been proved that *H. pylori* eradication can reduce the incidence of gastric cancer. Therefore, scholars believe that every region should understand the background of *H. pylori* drug resistance, especially for children, and select proper drugs to improve the eradication rate.

H. pylori mainly resides on the surface of human gastric mucosal epithelial cells and a gastric mucus layer. It has been pointed out that *H. pylori* can damage gastric mucosal epithelial cells mainly by producing vacuolating toxins and promote the occurrence of inflammation. The gene encoding vacA toxin is the vacA gene. The coding region of the vacA gene can be divided into gene signal region (*s* region, there are *s1* and *s2* alleles) and open reading frame intermediate region (*m* region, there are *m1* and *m2* alleles).[11] In addition, cytotoxin-associated genes cagA is another major virulence gene in the pathogenesis of *H. pylori*. Still, due to the limitation of the current technology, we mainly detected vacA genotype. Generally speaking, vacA *s1/m1* gene encodes a large number of toxin proteins, *s1/m2* encodes a moderate amount of toxin proteins, and *s2/m1* and *s2/m2* encode a lower amount of toxin proteins. In this study, the vacA *s1* gene was positive in almost all *H. pylori*-positive individuals, mainly *s1/m2* and *s1/m1*. This is consistent with the virulence dominant genotype reported by Hai et al,[12,13] suggesting that the pathogenicity of *H. pylori* in Jilin is strong and easy to cause clinical diseases; however, we did not find significant differences in the virulence genotypes between children and adults, which suggests that we should also pay attention to the infection of *H. pylori* in children.

A meta-analysis reported that the resistance rates of *H. pylori* to clarithromycin, metronidazole, and levofloxacin were 28.9, 63.8, and 28%, respectively.[14] Drug resistance of *H. pylori* varies in different areas. Our results showed that the resistance rate of *H. pylori* to clarithromycin was the highest in Jilin (86.7% in children and 60.0% in adults), followed by metronidazole and levofloxacin, the resistance rate of *H. pylori* to tetracycline and amoxicillin were low. The results are consistent with the situation of *H. pylori* resistance in China; however, the specific drug resistance rate is generally higher than the average

Table 5. The Relationship Between Helicobacter pylori Resistance and Gender, Disease Types

Variable	Clarithromycin	Metronidazole	Levofloxacin	Amoxicillin	Tetracycline
Male					
Children	20(66.7%)	7(23.3%)	9(30.0%)	2(6.7%)	2(6.7%)
Adults	12(30.0%)	10(25.0%)	5(12.5%)	1(2.5%)	4(10.0%)
Female					
Children	6(20.0%)	1(3.3%)	3(10.0%)	2(6.7%)	0(0)
Adults	12(30.0%)	3(7.5%)	7(17.5%)	1(2.5%)	1(2.5%)
Non-ulcer dyspepsia					
Children	13(43.3%)	5(16.7%)	7(23.3%)	3(10.0%)	2(6.7%)
Adults	19(47.5%)	10(25.0%)	9(22.5%)	1(2.5%)	5(12.5%)
Peptic ulcer					
Children	13(43.3%)	3(10.0%)	5(16.7%)	1(3.3%)	0(0)
Adults	5(12.5%)	3(7.5%)	3(7.5%)	1(2.5%)	0(0)

level of the domestic population. They were considering the small sample size, and RUT positive samples were included in this study.

In the early 1990s, standard triple therapy (PPI plus 2 antibiotics) was used to eradicate *H. pylori* with a success rate of 90%. However, with the increase of drug resistance, current studies have pointed out that the eradication rate of *H. pylori* in drug-resistant *H. pylori* infections has been less than 50%,[15] far from reaching the desired level. In view of the above situation, China's guidelines recommend Bismuth-containing quadruple therapy (PPI plus Bismuth plus 2 antibiotics) as the main empirical treatment for *H. pylori* infection in adults, and Bismuth-free triple therapy for children. According to the background study of *H. pylori* resistance in Jilin, amoxicillin is recommended as a first-line drug choice, especially in children, and for adults and children over 8 years of age, tetracycline is also recommended. Clarithromycin is not recommended as a first-line treatment. The resistance rate of metronidazole and levofloxacin has reached about 30%, which is not recommended for primary treatment and can be used as a remedial treatment.

More importantly, we found that there are some differences between children and adults in the drug resistance of *H. pylori*. For example, the drug-resistance rate of clarithromycin in children was significantly higher than that in adults, especially in children with peptic ulcer. This indicates that we should not select antibiotics for children according to the drug resistance of *H. pylori* in adults. In the future, we will increase the sample size to establish the background of drug resistance of *H. pylori* in children. In addition, there are relatively few drugs available for eradicating *H. pylori* in children, which also urges us to develop new therapeutic drugs as soon as possible.

Ethics Committee Approval: Ethics committee approval was received for this study from the First hospital of Jilin University. (Approval No: 2017-443).

Informed Consent: All patients came from Jilin and signed the informed consent.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept, Design, Supervision and Critical Reviews - P.X.; Materials - Z.J.; Data Collection and Analysis - H.M.; Writing and Literature Search- W.L.

Conflict of Interest: The authors have no conflicts of interest to declare.

Financial Disclosure: This work was supported by National Natural Science Foundation of China [grant numbers 81800539]

REFERENCES

1. Magalhães Queiroz DM, Luzza F. Epidemiology of helicobacter pylori infection. Helicobacter. 2006;11(suppl 1):1-5. [CrossRef]
2. Delport W., van der Merwe SW. The transmission of Helicobacter pylori: The effects of analysis method and study population on inference. Best Pract Res Clin Gastroenterol. 2007;21(2):215-236. [CrossRef]
3. Nagy P, Johansson S, Molloy-Bland M. Systematic review of time trends in the prevalence of helicobacter pyloriinfection in china and the USA. Gut Pathog. 2016;8(1):8. [CrossRef]

4. Backert S, Clyne M. Pathogenesis of helicobacter pylori infection. **Helicobacter**. *2011;16(suppl 1):19-25.* [CrossRef]
5. Saber T, Ghonaim MM, Yousef AR, et al. Association of Helicobacter pylori cagA gene with gastric cancer and peptic ulcer in saudi patients. **J Microbiol Biotechnol**. *2015;25(7):1146-1153.* [CrossRef]
6. Song ZQ, Zhou LY. Helicobacter pylori and gastric cancer: clinical aspects. **Chin Med J (Engl)**. *2015 November 20;128(22):3101-3105.* [CrossRef]
7. Franck C, Hoffmann A, Link A, et al. Prevalence of helicobacter pylori infection among blood donors in saxony-anhalt, germany-a region at intermediate risk for gastric cancer. **Z Gastroenterol**. *2017;55(7):653-656.* [CrossRef]
8. Shimizu T, Chiba T, Marusawa H. Helicobacter pylori-mediated genetic instability and gastric carcinogenesis. **Curr Top Microbiol Immunol**. *2017;400:305- 323.* [CrossRef]
9. Arkkila PE, Seppälä K, Kosunen TU, et al. Helicobacter pylori eradication as the sole treatment for gastric and duodenal ulcers. **Eur J Gastroenterol Hepatol**. *2005;17(1):93-101.* [CrossRef]
10. Fallone CA, Moss SF, Malfertheiner P. Reconciliation of recent Helicobacter pylori treatment guidelines in a time of increasing resistance to antibiotics. **Gastroenterology**. *2019;157(1):44-53.* [CrossRef]
11. Ranjbar R, Chehelgerdi M. Genotyping and antibiotic resistance properties of Helicobacter pylori strains isolated from human and animal gastric biopsies. **Infect Drug Resist**. *2018;11:2545-2554.* [CrossRef]
12. Almeida N, Donato MM, Romãozinho JM, et al. Correlation of Helicobacter pylori genotypes with gastric histopathology in the central region of a South-European country. **Dig Dis Sci**. *2015;60(1):74-85.* [CrossRef]
13. Alaoui Boukhris S, Benajah DA, El Rhazi K, et al. Prevalence and distribution of Helicobacter pylori cagA and vacA genotypes in the Moroccan population with gastric disease. **Eur J Clin Microbiol Infect Dis**. *2012;31(8):1775-1781.* [CrossRef]
14. Hu Y, Zhu Y, Lu NH. Primary antibiotic resistance of Helicobacter pylori in China. **Dig Dis Sci**. *2017;62(5):1146-1154.* [CrossRef]
15. Graham DY, Dore MP. Helicobacter pylori therapy: A paradigm shift. **Expert Rev Anti Infect Ther**. *2016;14(6):577-585.* [CrossRef]